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Steroid - DNA Conjugates: Improved Triplex Formation with 5-Amido-(7-Deoxycholic Acid)-dU incorporated Oligonucleotides

Dimpan Bhatia. Li Yue-Ming and Krishna N Ganesh*

Division of Organic Chemistry (Synthesis), National Chemical Laboratory, Pune 411008, INDIA

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Abstract The synthesis of oligodeoxyribonucleotides incorporating 7-deoxycholic acid conjugated at C5 of dU is described. When used as third strand, these form oligonucleotide triplexes with higher stability compared to unmodified controls at physiological pH.

Covalent conjugation of small molecule ligands such as intercalators, metal complexes and reporter groups to oligonucleotides are well known to modulate their biophysical and biochemical properties[1]. Some of the benefits of conjugation are (i) stabilization of DNA duplexes and triplexes by intercalators,[2] (ii) targeted cleavage of complementary strands in duplexes and triplexes by metal complexes,[3] and (iii) detection of DNA hybridization by fluorescent reporter groups.[4] The functional attributes imparted by the conjugated ligands to ODNs are also useful in the antisense/antigene therapeutic strategies[5] and in the development of DNA based diagnostics.[4] One of the serious limitations of the oligonucleotide based antisense/antigene therapeutic agents is their poor uptake by cells. Conjugation of a cholesteryl moiety to the 5' or 3' terminii through a short linker arm has recently been shown to enhance cellular uptake,[6] improve antisense efficacy,[7] alter tissue distribution[8] and improve the nuclease resistance.[9] The lipophilic steroid increases the affinity of ODNs for cell membranes and low density lipoproteins and resulted in improved antiviral properties by non-antisense mechanisms such as binding to key viral and cell surface proteins.[10] The steroid moiety upon conjugation to cordycepin exhibited enhanced HIV inhibition activity.[11]

The structure-activity relationships of 5'/3'-terminal cholesteryl conjugation to ODNs through a variety of linkers has indicated considerable dependence of cytotoxicity on the structure of linkage. While hydrophobicity at 3'-terminus favoured biological activity, that at the 5'-end was poorly tolerated and long linkers reduced or eliminated potency of anticancer activity studied in colon and breast carcinoma cell lines.[12] It has been shown that lipophilic ODN modifications such as cholesteryl present on both strands can increase the T_m of derived duplexes through mutual hydrophobic interactions on complementary strands.[13] In all the cases reported so far, the steroid conjugation was always at 5'-or 3'-terminus. In view of the above observations, it was thought worthwhile to synthesize ODN conjugates in which the steroid is located on the nucleobase which also enables multiple internal labelling. *Further, it would also be interesting to study the*

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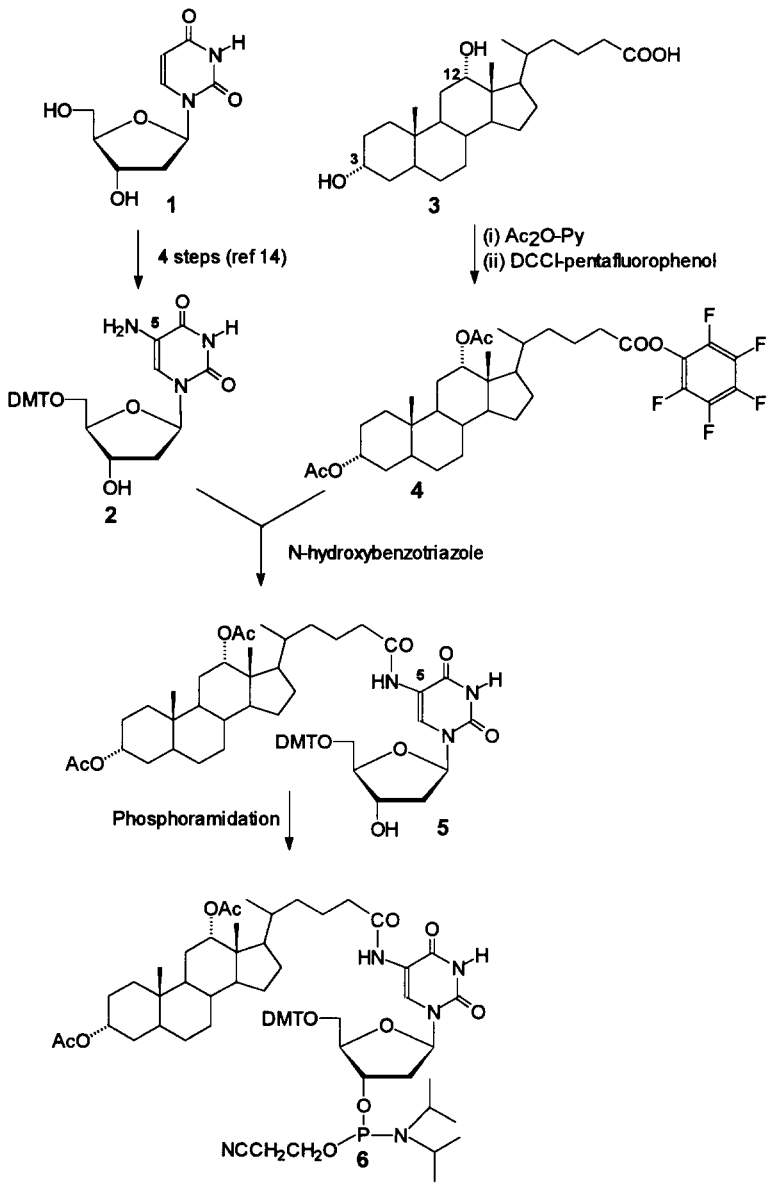
hitherto unknown structural effects of steroid conjugation on the triplex forming ability of ODNs. This report deals with synthesis of ODNs containing steroid conjugation at C5-position of nucleobase dU and it is demonstrated that these efficiently act as third strands in binding to complementary DNA duplex to form triplexes with improved stability as compared to unmodified controls. The choice of 7-deoxycholic acid as the steroid component was arbitrary due to its easy availability.



7	*5′*	TTC	TTT	TTT	CTT	TTT	TCT	*3′*	
8		TTC	XTT	TTT	CTT	TTT	TCT		
9		TTC	TTT	TTX	CTT	TTT	TCT		
10		TTC	TTT	TTT	CTT	TTT	XCT		
11		GCG	AAG	AAA	AAA	GAA	AAA	AGA	CGC
12		GCG	TCT	TTT	TTC	TTT	TTT	CTT	CGC
13			TCT	TTT	TTC	XTT	TTT	CTT	
14			TCT	TTT	TTC	TTT	TTT	CTT	

X=dU(5-NHCO-7-deoxycholic acid)

Synthesis of 7-deoxycholic acid-dU oligonucleotides

Oligodeoxynucleotides (**8-10,13**) carrying the 7-deoxycholic acid moiety were synthesized by substituting 5-amido-(7-deoxycholic)-2'-deoxyuridine for thymidine at predetermined positions in the oligonucleotide sequences. Starting from 2'-deoxyuridine **1** and following the published procedure[14] 5'-O-DMT-5-amino-2'-deoxyuridine **2** was sythesized in four steps. In a second series of reactions, 7-deoxycholic acid **3** was acetylated with acetic anhydride in pyridine and the resulting 3,12-diacetyl-7-deoxycholic acid was converted to its pentafluorophenyl active ester **4.** This was coupled with 5'-O-DMT-5-amino-uridine **2** to obtain the nucleoside-steroid conjugate **5**. The phosphitylation of the 3'-hydroxyl of this conjugate following standard procedures[15] gave the desired nucleoside 3'-phosphoramidite **6** in 84% yield. All compounds were purified by column chromatography and were found to be homogeneous by tlc and NMR spectroscopy.[16] The oligonucleotides **7-14** were synthesized on a Pharmacia Gene Assembler Plus synthesizer using the standard phosphoramidite chemistry with incorporation of the modified dU-7-deoxycholyl monomer (X) at the desired positions. The coupling efficiencies obtained with the modified monomer **6** were similar to those obtained with the standard phosphoramidites. All ODNs were deprotected and purified following standard procedures in oligonucleotide synthesis[17] and as indicated by their different retention times in FPLC, the cholesteryl moiety was retained in the modified ODNs **8-10** and **13**.

UV Melting Studies

The duplexes of the modified ODNs were constituted with the common unmodified complementary strand **11** and their thermal stability determined from temperature dependent UV absorbance. The modified duplex **13:11** T_m (52°C) was lower in comparison with that of control **14:11** (T_m, 57°C) under identical conditions of pH and salt concentration.[18] This is consistent with our previous observations on other C5-dU conjugated oligonucleotides which showed destabilization of duplexes.[14] The triplexes were constituted from equimolar amounts of either the unmodified ODN **7** (control) or deoxycholic acid modified strands **8,9** and **10**

and the duplex **11:12** in Tris buffer, pH 7-7.2, containing physiological concentrations of salts.[18] The biphasic transitions in melting profiles in Figure 1 show that the deoxycholic acid conjugated ODNs interact with the common duplex **11:12** to form triple helices. Significantly, the unmodified control strand, under identical conditions of pH and salt concentration forms a triplex with a much lower stability. It can be seen from Table 1 that compared to the melting of unmodified triplex **7*11:12** (entry 1), that of steroid conjugated triplexes (entry 2-4) are higher by 13-16°C. The melting data also indicate that triplexes with modification in middle of the strand (as in **9**) is less stable ($\Delta T_m = 3°$) compared to triplexes with modifications closer to the 3'/5' terminii (as in **8**, **10**). This is in agreement with general patterns of stability as a function of position of modifications observed in other cases.[19] Significantly all modified triplexes were relatively more stable than the control triplex. It is also seen from Figure 1 that the duplex T_m in all triplexes including that of control, does not show any variation, since it is common to all of them.

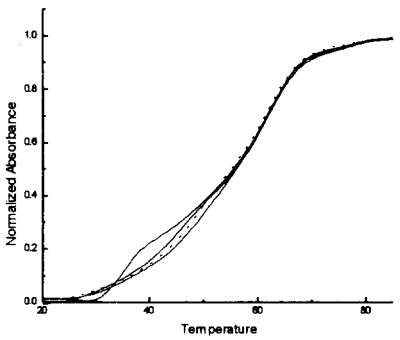


Figure 1: UV-melting curves for triplexes (a): **7*11:12**, (b) **9*11:12**, (c) **8*11:12** and (d) **10*11:12**

Table 1: UV-T_m (°C) data for triplexes*

	Triplex	T_m(°C)
1	**7*11:12**	33
2	**8*11:12**	49
3	**9*11:12**	46
4	**10*11:12**	49

Recently, we have shown that ODNs containing spermine conjugation at N^4 of cytosine exhibit enhanced triplex stability resulting from a faster reassociation of third strand due to electrostatic interaction of the cationic appendage with the corresponding duplex, evidenced by a lack of hysteresis in melting curve under cooling conditions.[19] In case of steroid-ODN conjugates, considerable hysteresis for the triplex⇔duplex transition was seen similar to control triplexes, indicating that the observed stability is not a result of improved reassociation of strands. It is possible that hydrophobic interactions arising from the large steroid ring positioned in the major groove of duplex may contribute to binding. The fact that triplex to duplex transition in modified ODNs is less sharp than the natural one suggests the origin of stabilization to be entropic in

nature. Alternatively, the hydroxy groups on steroid ring may be involved in specific hydrogen bonding with bases and from present the results, it is difficult to delineate the origin and extent of these contributions. Replacing the hydroxy functions on conjugated steroid component by amino groups (steroidal polyamines) may lead to additional stabilizing electrostatic interactions[20] and such work is in progress.

In summary, this communication demonstrates that covalent attachment of a steroid component such as 7-deoxycholic acid to nucleobase dU in the third strand of DNA triplex enhances the stability of derived triplexes. Since steroids can assist in the interaction with membranes to modulate biological activity, the present observations may have implications for design of efficient bioconjugates for transport of ODNs across the cellular membranes for antigene activity via triplex formation.

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16. Selected Data: Compound **6**, ^{1}H NMR ($CDCl_3$) δ: 7.75-7.2 (m,,10 H, H6 and ArH DMT), 6.8 (m, 4H, ArH DMT), 6.35 (t, 1H, H1'), 4.7 (m, 1H, H3'), 4.35 (m, 2H, H5',5"), 3.99 (m,1H, H4'), 3.8 (s, 6H, 2 OCH_3 DMT), 2.3 (m, 2H, H2',2"), 2.1 (s, 3H,$COCH_3$ Ac), 2.05 (s, 3H, $COCH_3$ Ac), 0.9 (s, 3H, C19-CH_3 Chol), 0.81 (d, 3H, C21-CH_3 Chol), 0.7(s, 3H, C18-CH_3 Chol); Compound **7**, ^{31}P NMR ($CDCl_3$) δ : 150.2 and 149.9 ppm.
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18. Duplexes were constituted by mixing equimolar amounts of the appropriate strands in buffer containing sodium cacodylate (pH 7.0, 10 mM), $MgCl_2$ (10 mM) and NaCl (100 mM). The strands were heated at 80°C for 5 min. followed by slow cooling. The annealed samples were maintained at 4°C for 1 hr. before being subjected to melting studies All triplexes were similarly constituted by mixing equimolar amounts of the appropriate strands in a buffer containing 25mM Tris, 100 mM NaCl, 20 mM $MgCl_2$ at pH 7.0. All melting experiments were carried out by recording UV absorbance at 260 nm within the temperature range 20-85°C at a heating rate of 0.5°C per minute. The melting temperatures were obtained by determining the points of inflection of the absorbance vs temperature curves using first derivative plots.
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